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immobilized nucleic acid and its availability for hybridization are unknown. When using probes that have the capacity to self-anneal (e.g., nick-translated double-stranded DNA), the following rule of thumb is useful: Allow the hybridization to proceed for a time sufficient to enable the probe in solution to achieve $1–3 \times C_o t_{1/2}$. In 10 ml of hybridization solution, 1 μg of a probe of 5-kb complexity will reach $C_o t_{1/2}$ in 2 hours. To determine the time of half-renaturation for any other probe, simply enter the appropriate values into the following equation:

$$1/x \times y/5 \times z/10 \times 2 = \text{number of hours to achieve } C_o t_{1/2}$$

where x = the weight of the probe added (in micrograms), y = its complexity (for most probes, complexity is proportional to the length of the probe in kilobases), and z = the volume of the reaction (in milliliters).

After hybridization to $3 \times C_o t_{1/2}$ has been reached, the amount of probe available for additional hybridization to the filter is negligible. For probes that do not have the capacity to self-anneal (e.g., RNA probes synthesized in vitro by bacteriophage-encoded DNA-dependent RNA polymerases; see Chapter 10), the hybridization time may be shortened, since the lack of a competing reaction in the solution favors hybridization of the probe to the DNA immobilized on the filter.

5. Several different types of agents can be used to block the nonspecific attachment of the probe to the surface of the filter. These include Denhardt's reagent (Denhardt 1966), heparin (Singh and Jones 1984), and nonfat dried milk (Johnson et al. 1984). Frequently, these agents are used in combination with denatured, fragmented salmon sperm or yeast DNA and detergents such as SDS. In our experience, virtually complete suppression of background hybridization is obtained by prehybridizing filters with a blocking agent consisting of 5 × Denhardt's reagent, 0.5% SDS, and 100 μg/ml denatured, fragmented DNA. We recommend this mixture whenever the signal-to-noise ratio is expected to be low, for example, when carrying out northern analysis of low-abundance mRNAs or Southern hybridizations with single-copy sequences of mammalian DNA. However, in most other circumstances (Grunstein/Hogness hybridization [1975], Benton/Davis hybridization [1977], Southern hybridization [1975] of abundant DNA sequences, etc.), we recommend using 0.25% nonfat dried milk (0.05 × BLOTTO; Johnson et al. 1984). This is much less expensive, easier to use than Denhardt's reagent, and, for these purposes, gives results that are equally satisfactory. In general, Denhardt's reagent is more effective for nylon membranes. The signal-to-noise ratio obtained with most brands of nylon membranes is higher with Denhardt's reagent than with BLOTTO. Nonfat dried milk is not recommended when using RNA probes or when carrying out northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase activity. For more information about blocking agents, see Table 9.1.

6. Blocking agents are usually included in both the prehybridization and hybridization solutions when nitrocellulose filters are used. However, when the nucleic acid is immobilized on nylon membranes, the blocking agents are often omitted from the hybridization solution, since high

TABLE 9.1 Blocking Agents Used to Suppress Background in Hybridization Experiments

Agent	Recommended uses
Denhardt's reagent	northern hybridizations hybridizations using RNA probes single-copy Southern hybridizations hybridizations involving DNA immobilized on nylon membranes

Denhardt's reagent (Denhardt 1966) is usually made up as a 50× stock solution, which is filtered and stored at −20°C. The stock solution is diluted tenfold into prehybridization buffer (usually 6× SSC or 6× SSPE containing 0.5% SDS and 100 μg/ml denatured, fragmented salmon sperm DNA). 50× Denhardt's reagent contains 5 g of Ficoll (Type 400, Pharmacia), 5 g of polyvinylpyrrolidone, 5 g of bovine serum albumin (Fraction V; Sigma), and H_2O to 500 ml.

BLOTTO	Grunstein/Hogness hybridization Benton/Davis hybridization all Southern hybridizations other than single-copy dot blots

1× BLOTTO (Bovine Lacto Transfer Technique Optimizer; Johnson et al. 1984) is 5% nonfat dried milk dissolved in water containing 0.02% sodium azide. It should be stored at 4°C and diluted 25-fold into prehybridization buffer before use. BLOTTO should not be used in combination with high concentrations of SDS, which will cause the milk proteins to precipitate. If background hybridization is a problem, NP-40 may be added to the hybridization solution to a final concentration of 1%. BLOTTO should not be used as a blocking agent in northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase.

Caution: Sodium azide is poisonous. It should be handled with great care, wearing gloves, and solutions containing it should be clearly marked.

Heparin	Southern hybridization in situ hybridization

Heparin (Sigma H-7005 porcine grade II or equivalent) is dissolved at a concentration of 50 mg/ml in 4× SSPE or 4× SSC and stored at 4°C. It is used as a blocking agent at a concentration of 500 μg/ml in hybridization solutions containing dextran sulfate; in hybridization solutions that do not contain dextran sulfate, heparin is used at a concentration of 50 μg/ml (Singh and Jones, 1984).

Denatured, fragmented salmon sperm DNA	Southern and northern hybridizations

Salmon sperm DNA (Sigma type III sodium salt) is dissolved in water at a concentration of 10 mg/ml. If necessary, the solution is stirred on a magnetic stirrer for 2–4 hours at room temperature to help the DNA to dissolve. The concentration of NaCl is adjusted to 0.1 M, and the solution is extracted once with phenol and once with phenol:chloroform. The aqueous phase is recovered, and the DNA is sheared by passing it 12 times rapidly through a 17-gauge hypodermic needle. The DNA is precipitated by adding 2 volumes of ice-cold ethanol. It is then recovered by centrifugation and redissolved at a concentration of 10 mg/ml in water. The OD_{260} of the solution is determined and the exact concentration of the DNA is calculated. The solution is then boiled for 10 minutes and stored at −20°C in small aliquots. Just before use, the solution is heated for 5 minutes in a boiling-water bath and then chilled quickly in ice water. Denatured, fragmented salmon sperm DNA should be used at a concentration of 100 μg/ml in prehybridization solutions.

concentrations of protein are believed to interfere with the annealing of the probe to its target. This quenching of the hybridization signal is particularly noticeable when oligonucleotides or probes less than 100 nucleotides in length are used.

7. In the presence of 10% dextran sulfate or 10% polyethylene glycol, the rate of hybridization is accelerated approximately tenfold (Wahl et al. 1979; Renz and Kurz 1984; Amasino 1986) because nucleic acids are excluded from the volume of the solution occupied by the polymer and their effective concentration is therefore increased. Although dextran sulfate and polyethylene glycol are useful in circumstances where the rate of hybridization is the limiting factor in detecting rare sequences (e.g., northern or genomic Southern blots), they are of no benefit when screening bacterial colonies or bacterial plaques. In addition, they can sometimes lead to high backgrounds, and hybridization solutions containing them are always difficult to handle because of their viscosity. We therefore recommend that dextran sulfate and polyethylene glycol not be used unless the rate of hybridization is very slow, the filter contains very small amounts of DNA, or the amount of radiolabeled probe is limiting.

8. To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength (6 × SSC or 6 × SSPE) at a temperature that is 20–25°C below the melting temperature (T_m). Both solutions work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, 6 × SSPE is preferred because of its greater buffering power.

9. In general, the washing conditions should be as stringent as possible (i.e., a combination of temperature and salt concentration should be chosen that is approximately 12–20°C below the calculated T_m of the hybrid under study). The temperature and salt conditions can often be determined empirically in preliminary experiments in which samples of genomic DNA immobilized on filters are hybridized to the probe of interest and then washed under conditions of different stringencies.

10. To minimize background problems, it is best to hybridize for the shortest possible time using the minimum amount of probe. For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 μg of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater) should be used and hybridization should be carried out for 12–16 hours at 68°C in aqueous solution or for 24 hours at 42°C in 50% formamide. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater).

11. *Useful facts*:

    a. The T_m of the hybrid formed between the probe and its target may be estimated from the following equation (Bolton and McCarthy 1962):

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The use of heparin as a simple cost-effective means of controlling background in nucleic acid hybridization procedures

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ABSTRACT

The use of heparin as a simple, effective and cheap substitute for conventional methods of controlling background in hybridization procedures is described and illustrated with reference to the use of DNA probes in filter and in situ hybridization. Possible specific mechanisms for this heparin effect are discussed.

INTRODUCTION

Refractory background radioactivity in nucleic acid filter hybridization varies in severity with different types of probes, annealing conditions and filter membranes. Background problems are also encountered in in situ hybridization where they are possibly even more intractable because of the unavoidable complexity in the substrate and the fact that vigorous non-specific methods of control, such as Denhardt's (1) solution cannot be used without damage to the cytological structure.

The possibility of a relatively specific means of background control in in situ hybridization was suggested by Maitland et al (2). These workers described non-hybridization binding of labeled DNA probes which was specific to certain cell types. Such binding was substantially reduced by the inclusion in the annealing reaction of the polyanionic glycan, heparin but the optimal conditions for this effect were not determined.

Heparin has a substrate binding affinity similar to that of DNA, especially with respect to certain proteins (3). Furthermore, it can abolish the binding of proteins which increase the affinity of DNA for nitrocellulose membranes by specifically combining with them (4,5). Given this well-characterised property, it is surprising that the possible use of heparin controlling non-specific substrate binding of DNA in both filter and in in situ hybridization apparently has not been explored previously. Accordingly we have examined this aspect and have shown that the inclusion of heparin in the hybridization annealing solution alone can dispense entirely with the use of Denhardt's solution and carrier DNA. This modification results in the consistent and complete abolition of background in both filter and in situ hybridization under a variety of different conditions. Moreover the modified hybridization protocol is much simpler, faster and

also considerably cheaper than existing methods.

MATERIALS AND METHODS

DNA extraction.

DNA from livers of male and female mice was isolated by homogenizing the tissue in TES buffer (30mM Tris, 5mM EDTA, 50mM NaCl, pH.8.0), lysing the cells with sarkosyl NL30 (Geigy) and by isopycnic centrifugation in CsCl-ethidium bromide gradients as previously described (6).

DNA restriction and gel electrophoresis.

Restriction enzymes and DNA polymerase were obtained from Boehringer Mannheim, New England Biolabs and Bethesda Research Laboratories. DNA was digested by incubating at a ratio of 1µg DNA to 2 units of enzyme for 4-5h in the buffers and conditions specified by the manufacturer. 0.6 to 1% agarose gels were run in a vertical floating gel apparatus at 30V for 11-12h in a buffer containing 15mM Tris, 18mM NaH_2PO_4 and 0.5mM EDTA, pH 7.8.

Electrophoretic transfer of nucleic acids.

Electrophoretic transfer of gel-fractionated DNA to nitrocellulose sheets (7) was carried out using a Hoeffer transfer unit after depurination (8). DNA restriction fragments were transferred from 2.5 mm thick agarose slab gels by the following procedure: the gel was gently shaken for 15 min in 250 ml of 0.25M HCl, washed gently with distilled water to remove the residual acid, denatured in two changes 15 min each of 250 ml 0.2M NaOH and 0.6M NaCl and neutralized in 3 changes each of 500 ml of transfer buffer (TB; 0.025M Na_2HPO_4/NaH_2PO_4, pH 6.5). The transfer was carried out in TB at room temperature for 1h at 27V keeping the unit cool with circulating cold tap water. The efficiency of transfer was assessed comparatively using the following membranes: Schleicher and Schull membrane filters Ref.No. 402097, pore size 0.1 um; Gene Screen hybridization transfer membrane Cat.No. NEF-972, Lot No. 354 GS21; Pall Biodyne transfer membranes P/N BNNG3R, rating 1.2 micron, Lot No. 172340. After transfer, the membranes were carefully washed with TB to remove residual agarose, dried at room temperature and baked at 80°C for 2-3h.

Blot hybridization.

The following stock solutions were prepared: 20x SET (0.5M NaCl, 0.03M Tris, 2mM EDTA, pH 7.4); 50% W/V sodium dextran sulphate aqueous solution, stored at 4-5°C; 50 mg/ml heparin sodium salt grade II from porcine intestinal mucosa (Sigma,H-7005) in 4xSET, stored at 4-5°C for several months; 20% W/V sodium pyrophosphate; 20% W/V sodium dodecyl sulphate (SDS).

i) Hybridization without dextran sulphate: The filter was first soaked in 4xSET and placed in a plastic bag with 10ml of hybridization solution per 10x14cm membrane. Hybridization salt solution contained 4xSET, 0.1% sodium pyrophosphate solution, 0.2% SDS, 50 µg/ml heparin. To avoid the formation of air bubbles the solution was preheated to 65°C before use. After heat sealing, the bag was placed in a shaking distilled water bath and incubated with vigorous shaking at 65°C for 2-3h. During this time the hybridization

bag was kept submerged by another water-filled plastic bag. The denatured, or single-stranded probe was then added to the bag without introducing air bubbles. The bag was then resealed and the solution throughly mixed. The bag was returned to the water bath at 65°C and incubation with vigorous shaking continued for 4-15h depending on the desired C_oT. For convenience, incubation was normally continued overnight.

ii) Hybridization in the presence of dextran sulphate. The procedure is basically the same as described above except that the solution contained 10% dextran sulphate and 10x the concentration of heparin (500µg/ml).

iii) Washing membrane filters. After either hybridization procedure, the filter was removed from the hybridization bag, briefly dipped in 2xSET at room temperature, re-sealed in a fresh plastic bag with 400-500ml of prewarmed wash buffer (2xSET,0.2% SDS) and vigorously agitated for 30min at 65°C. The wash buffer was replaced with prewarmed fresh buffer and the step repeated. In the third, and final, washing the wash buffer was replaced with prewarmed buffer adjusted in SET concentration so as to achieve the desired stringency, in the work described here 1xSET, 0.2% SDS, and agitated as before. Finally, the membrane was rinsed in 2xSET at room temperature, air dried and autoradiographed. Filters can be re-used after removal of the hybridized probe by washing in 0.1% wash buffer (1x wash buffer contains 50mM Tris-HCl, pH 8.0, 2mM EDTA, 0.5% sodium pyrophosphate) for 2h at 65°C.

Probe labeling.

Double-stranded DNA was labeled by nick-translation according to the method of Maniatis et al.(9), with minor modifications, using ^{32}P-dCTP (sp.act. 410 Ci/mM,) or ^{32}P-dATP (sp.act. 3000 Ci/mM, Radiochemical Centre, Amersham). The reaction was stopped by adding 5µl of 0.5M EDTA (in the total incubation volume of 20µl). 10-20µg of E.coli carrier DNA was then added and the DNA precipitated with 60µl of absolute ethanol (2 x volume) at -20°C for 2h or -70°C for 30min. Unincorporated ^{32}P dCTP was removed by 3-4 washes in absolute ethanol in a microcentrifuge. Finally, the precipitate was lyophilised, dissolved in 0.2ml of 0.1xSET, denatured for 20min in boiling water, cooled rapidly on ice and used immediately or stored at -20°C. Occasionally, we have used a nick-translated probe without first removing the unincorporated ^{32}P dCTP without experiencing any autoradiographic background problems.

Preparation of single-stranded DNA probes.

Single stranded DNA probes were prepared according to the method of Hu and Messing (10). A 16 base primer CACAATTCCACACAAC complementary to the region 5' to the multiple cloning sites of M13mp7, M13mp8 and M13mp9 (New England Biolabs.) was used to initiate limited downstream synthesis of part of the complementary strand of the M13 sequence, leaving the cloned insert single-stranded. This partially single stranded probe labeled with ^{32}P dATP (sp.act. 3000 Ci/mM) had a specific activity of $3x10^8$ cpm/µg.

Cytological preparations.

Microscope slide preparations were made from highly concentrated drops of mouse spermatozoa recovered by squeezing the dissected vas deferens in 0.9%

NaCl solution. In some samples, the sperm were treated with hypotonic 0.075M KCl solution for 8 min at 37°C. Smears were fixed in 3:1 methanol-acetic acid for 30 min, air dried and stored at 4°C.

In situ hybridization.

To make the sperm nuclei accessible to the probe, the slides were incubated in 50mM dithiothreitol (DTT) in 0.1M Tris-HCl buffer, pH 8.0 at room temperature for 20 min., rinsed in 2xSSC (SSC is 0.15M NaCl, 0.015M sodium citrate, pH 7.0), dehydrated through an (50%-100%) alcohol series and air dried. To remove histone and non-histone proteins, the slides were immersed in a solution containing dextran sulphate (2mg/ml, Pharmacia), heparin (0.2mg/ml, Sigma), 10mM Tris (pH 9.0), 10mM EDTA and 0.1% Nonidet P-40 at 5°C for 45 min, rinsed through two 15 min changes of 2xSSC , dehydrated through an ethanol series ,air dried and heat denatured (6). Air dried slides were rinsed briefly in heparin solution (50ug/ml in H_2O) and air dried without further rinsing. The nick translated DNA probe was denatured, lyophilised and diluted to the desired volume and salt concentration immediately before use. The hybridization mix contained nick-translated DNA (uncloned Bkm; Bkm clone 2(8), or lambda phage DNA), 1.7 ug/ml, sp.act. between $2x10^7$ and $4x10^7$ cpm/ug; 3xSSC; 10% dextran sulphate and 500 ug/ml heparin. No carrier DNA was used. Where dextran sulphate was not used the concentration of heparin was reduced to 50 ug/ml. For in situ hybridization the general procedure of Jones (11) was followed with minor modifications. 6 ul of hybridization mix was used on each slide and annealing was carried out at the T_{opt} (60°C) for 4 hr. The hybridized slides were washed in 2xSSC at 60°C for 1h., washed overnight in 2xSSC at 4°C, passed through an ethanol series and air dried. Hybridized slides were dipped in Ilford K2 nuclear emulsion, exposed for 1 to 10 weeks, developed in Kodak D19b developer, fixed in Ilford Hypam, stained in Giemsa and photographed as described previously (12).

RESULTS.

Blot hybridization.

Heparin concentrations over the range of 5 to 1000µg/ml were tested in order to establish the minimum concentration effective in abolishing background in the absence of other measures. As might be expected, the higher the effective concentration and the specific activity of the probe the more heparin needed to effectively supress background.At the probe specific activities achieved ($5x10^7$-$3x10^8$ cpm/µg),and with added probe of from $2x10^5$-$2x10^6$ cpm/ml,it was found necessary to add 50-200 µg/ml of heparin in the absence of dextran sulphate. This was required to be increased to 500-700 µg/ml of heparin in the presence of dextran sulphate. The tenfold higher concentration presumably is necessary to offset the concentrating effect of dextran sulphate on the probe. To obtain complete control of background it was found effective to include heparin only in the hybridization mix. Hybridization in dextran sulphate of the single-stranded cloned Bkm probe 2(8) (13) to an AluI digested mouse DNA blot is shown in Fig 1. with and

Figure 1. 6µl of male and female mouse genomic DNA digested with AluI, fractionated on 1% agarose gel, transferred onto Schleicher & Schull membrane filters and hybridized with the ^{32}P single-stranded probe 2(8) $6x10^5$ cpm/ml (specific activity $3.2x10^8$ cpm/µg) for 12h at 65°C (see Experimental Procedures) exposed for 15h without pre-sensitizing the film. Note absence of background in panel 1 which was hybridized in the presence of dextran sulphate and heparin. Panel 2 shows the same sequences hybridized under identical conditions in the absence of heparin. Note patchy background.

without 500µg/ml of heparin (panels 1 and 2 respectively). Heavy patchy background occurs only in the filter hybridized in the absence of heparin. Hybridization to low copy number sequences in mouse and yeast genomic DNA using the heparin/dextran sulphate method is shown in Fig.2 . Panel 1 shows EcoR1 digested male and female mouse DNA hybridized with a probe of a mouse male-specific fragment (M34) recovered from a mouse genomal library. The absence of background is evident from the female DNA track which lacks the sequence in question. Panel 2 shows yeast DNA hybridized with a cloned MAT sequence probe (2DX) and is, similarly, free from non-specific background.

A brief comparative assessment of the heparin and Denhardt methods was made

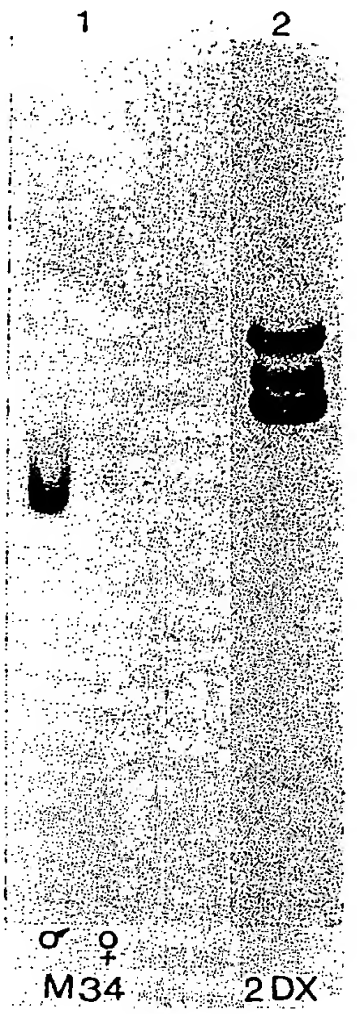

Figure 2. Hybridization of cloned probes of single copy sequences to genomic DNA in the presence of heparin and dextran sulphate showing absence of background.Panel 1: 6µg of EcoR1 digested male and female mouse DNA transferred and hybridized for 15h at 65°C with a male- specific ^{32}P labeled (specific activity $4x10^5$ cpm/µg) nick-translated phage lambda clone M34. Exposure time 3 days. Panel 2: 10µg of Hind 111 digested Saccharomyces cerevisiae genomic DNA transferred and hybridized for 15h at 65°C with ^{32}P-labeled (specific activity $2x10^8$ cpm/µg) nick-translated MAT clone 2XDX. Exposed for 20h.

by hybridizing duplicate AluI blots of mouse DNA using either Denhard's solution, as conventionally employed, or heparin as described here, under otherwise uniform conditions. It was found that heparin gave a comparatively cleaner background and a subjectively stronger signal, although this may not be apparent from the illustration (Fig.3). As might be expected, hybridization in the presence of Denhard's solution and added carrier DNA combined with heparin did not materially affect the result.

To test whether the heparin method works equally well using various commercially available filter membranes, AluI restricted male and female mouse DNA was electrophoretically transferred under identical conditions to three different examples of filter membrane (Schleicher & Schull, Gene Screen and Pall Biodyne). These were hybridized in the same annealing reaction with a single-stranded probe (referred to as 2(8)) which is quantitatively male-specific (13). The result is shown in Fig.4 in which

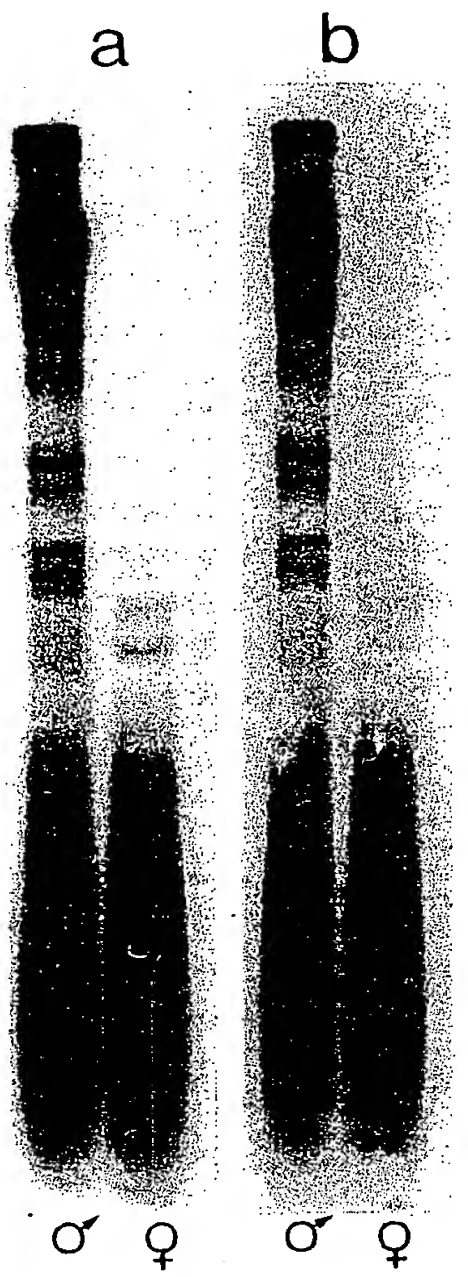

Figure 3. Southern transfer of AluI digested male and female mouse genomic DNA (6 µg each) onto Schleicher & Schull membrane, hybridized at 65°C with ^{32}P single-stranded clone 2(8) probe, 2x10^{5} cpm/ml (sp.act. 9x10^{7} cpm/µg) using two different methods: (a).Hybridized in the presence of heparin and dextran sulphate as described in the text. (b). Prehybridized overnight in 10xDenhardt solution (10x is 0.2% each of bovine serum albumin, Ficoll, and polyvinylpyrolidone), 0.1% sodium pyrophosphate, 0.2% SDS, 4xSET, 10% dextran sulphate and 100 µg/ml sheared denatured E.coli DNA). The hybridization mix contained 4xDenhard's solution together with the probe [as in (a)] but was otherwise identical to the prehybridization solution. Hybridization was for 12h at 65°C and washing as described in the text.

panels 1 and 2 refer to Schleicher & Schull membrane, respectively with, and without, DNA depurination prior to transfer. Panels 3 and 4 refer, respectively, to Gene Screen and Pall Biodyne; the DNA being depurinated prior to transfer. There was no non-specific background on any of the three filter types. It is apparent that depurination, as expected, increased the transfer efficiency of high molecular weight fragments. However, surprisingly, there was a very striking difference in the hybridization signal strength obtained with the filters from different manufacturers. This

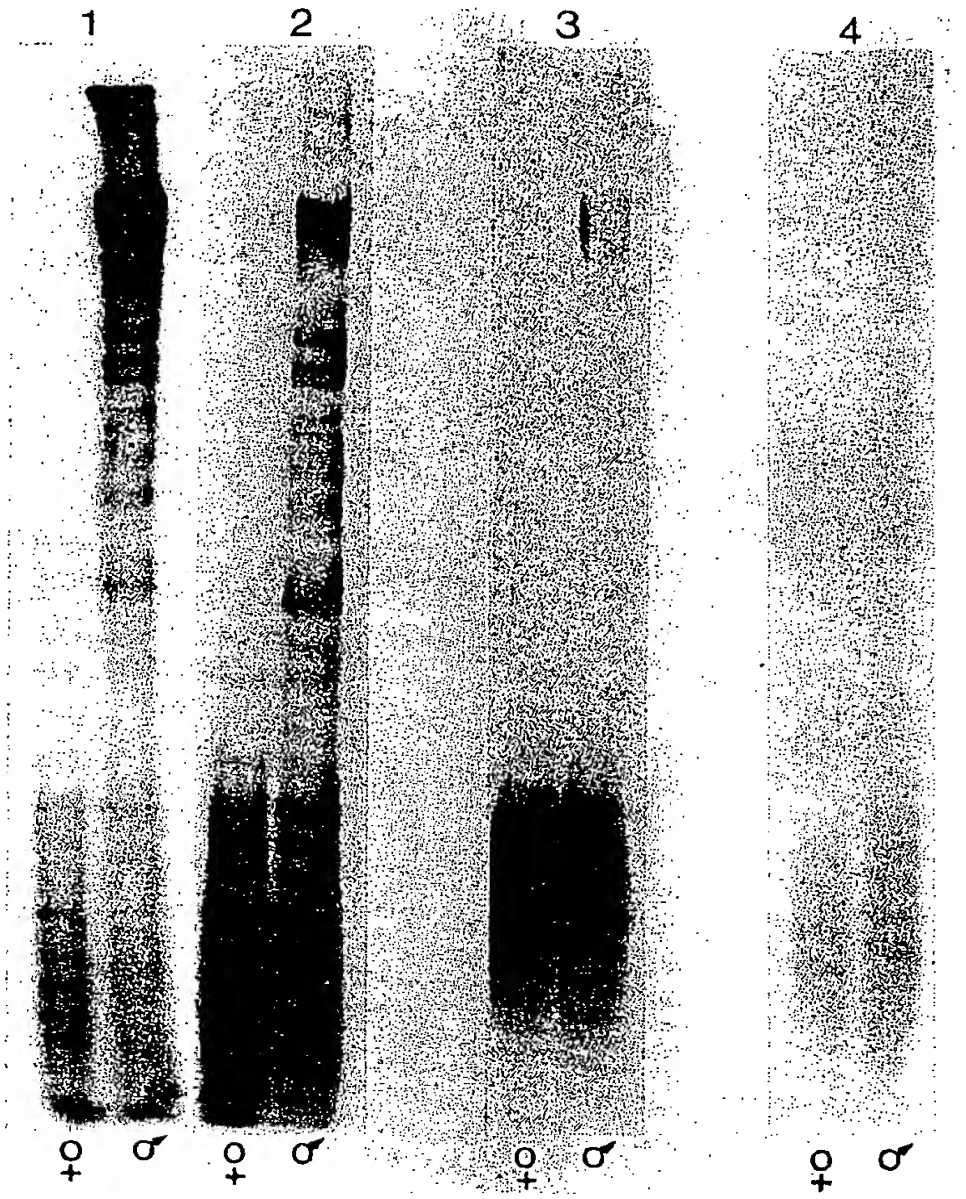

Figure 4. 6µg of Alu 1 digested male and female mouse genomic DNA fractionated on a 1% agarose gel which was then cut into strips and transferred under identical conditions onto the following filter membranes for hybridization. Panel 1: Schleicher & Schull filter membrane after HCl depurination of the DNA; Panel 2: Schleicher & Schull filter membrane without depurination; Panel 3: Gene Screen hybridization transfer membrane after HCl depurination; Panel 4:Pall Biodyne transfer membrane after HCl depurination. All filters were hybridized collectively with single-stranded ^{32}P labeled (specific activity $3x10^8$ cpm/µg) 2(8) probe, $1x10^6$ cpm/ml. The hybridization mixture contained 4xSET, 0.1% sodium pyrophosphate, 0.2% SDS, 50µg/ml heparin and $1x10^6$ cpm/ml of the probe. No dextran sulphate was used. Hybridization was at 65°C for 18h. Filters were exposed for 24h. Note absence of background but differences in hybridization signal strength between the filter samples which is highest on Schleicher & Schull. Retention of high molecular weight DNA fragments is relatively poor on Gene Screen and poorest on Pall Biodyne transfer membrane.

was not due to the use of heparin but appears to reflect differences in the efficiency with which high molecular weight DNA was retained by the different membranes under our conditions. The experiments were then repeated using the transfer method of Southern (21) with the same result, thus ruling out the possibility that such differential retention was due to the electroblotting procedure. However, electroblotting was more efficient than the Southern

procedure. These experiments were not designed to investigate this point but this result was highly repeatable between different samples of these filters. The sample which performed least well (Pall Biodyne) does not show the sex difference which is obvious on the most efficient sample (Schleicher & Schull) after the shorter exposure time. However, equivalent autoradiographs were obtained when the membranes having the poorer performance were exposed for 5 times as long, as shown in Fig.4. Although each manufacturer specifies different conditions for efficient transfer of DNA, which our experiments were not designed to compare, our subsequent experience is that comparable differences in efficiency between these filter samples were still evident when the manufacturer's recommendations are observed. Gene Screen Plus^{TM} however, was subsequently found to be markedly superior to Gene Screen in terms of the efficiency of DNA transfer and comparable in this respect with the performance of Schleicher & Schull membranes. According to the manufacturer's recommendations Gene Screen Plus^{TM} does not require Denhardt's treatment and we have not yet fully investigated its use in the heparin method.

In situ hybridization.

In situ annealing of sperm smears with nick translated uncloned Bkm, with single-stranded 2(7) or 2(8) probes (13) or with control probes of pBR322 or phage lambda DNA, revealed consistent and intense non-specific binding to sperm heads. Other cell types present in the smears did not show such binding (Fig.5a). Deproteinization of the smears (see materials & methods) was ineffective in reducing this non-specific binding. However, it was abolished by the inclusion of heparin in the hybridization procedure. The optimal concentrations of heparin as established by blot hybridization were employed. Thus, the denatured sperm preparations were pretreated in 50µg/ml of heparin and either 50µg/ml or 500µg/ml of heparin was included in the hybridization mix depending,respectively, on whether or not dextran sulphate was used (Fig.5b,c,d). In these preparations hybridization to specific regions of a proportion of the sperm nuclei can be seen against a background of hybridization (arrows). This probably reflects the fact that Bkm sequences are preferentially localised and concentrated in the proximal sex-determining region of the mouse Y chromosome (6,13,14,15,16,,17,). Thus, by this approach it may prove possible cytologically to differentiate Y- and X-bearing mouse sperm. In the preparations shown in Fig.5 some sperms have remained unlabeled (Fig.5C). The source of this effect is unknown but it might be due to variability in the thermal denaturation of DNA in individual sperm. All three Bkm-related probes described in this work gave similar results.

DISCUSSION

The discovery of Nygaard & Hall (18) that nitrocellulose filters strongly adsorbed single-stranded DNA, along with any hybridized RNA, led to the filter hybridization method in which DNA was irreversibly fixed to such

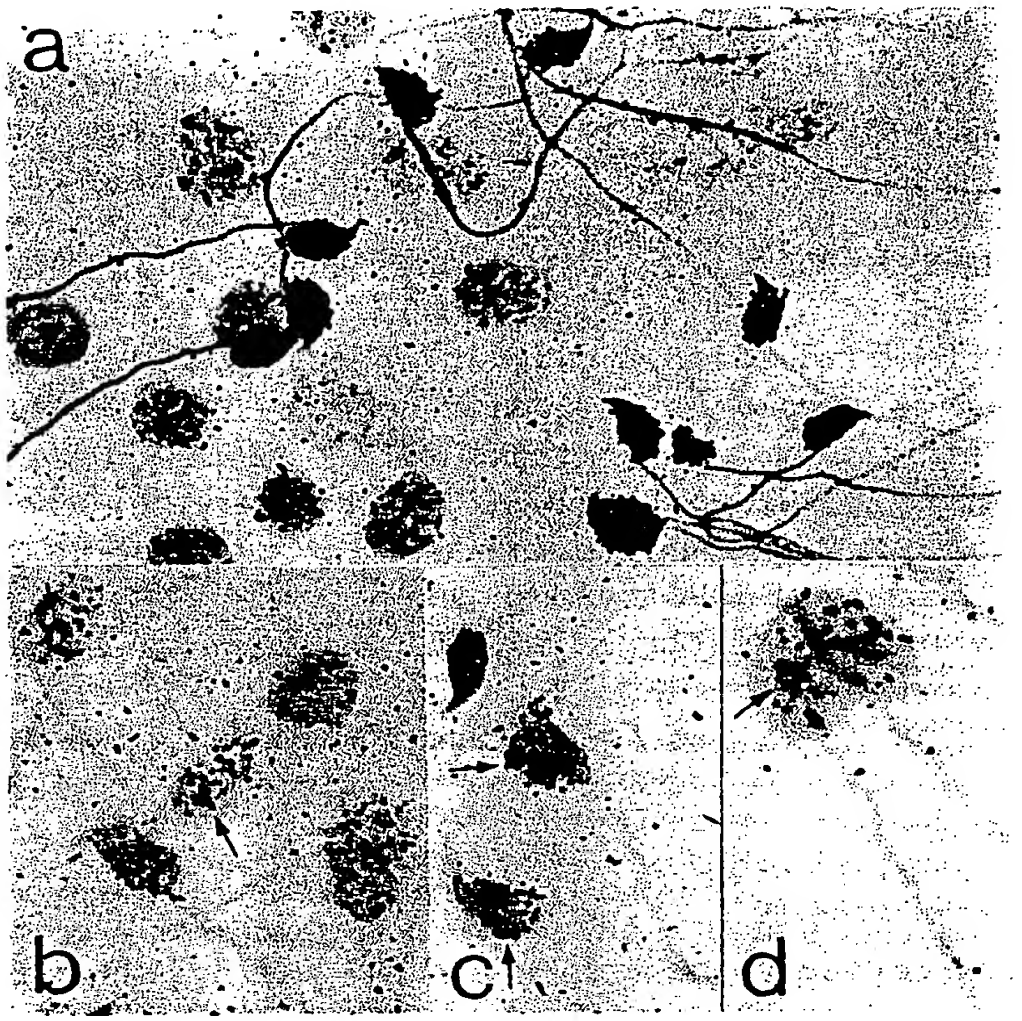

Figure 5. Mouse sperm smears hybridized in situ with ^{3}H-labeled, nick-translated uncloned Bkm probe as described in the Methods section. (a) Note non-specific binding of probe to the entire sperm heads but not to other cell types in the absence of heparin. 10 days exposure. (b) as in (a) but with heparin in the hybridization mix. Note hybridization now restricted to sperm head nucleus some of which show high concentrations of grains in a localized region of the nucleus (arrow) which may indicate the presence of the Y chromosome.(c) as in (b) but hybridized with single-stranded probe 2(8). arrows: sperms with concentrations of hybridization. (d) as in C showing two sperms side by side differing in hybridization pattern. 6 weeks exposure.

membranes before hybridization by drying at moderate temperatures (19). Denhardt (1) found that the non-specific adsorbtion of single-stranded DNA was prevented, without blocking specific hybridization, by pre-incubating the filters in 0.02% each of Ficoll, polyvinylpyrrolidone and bovine albumin in 3xSSC. He pointed out, however, that concentrations of bovine albumin above 0.05% reduced the specific binding of DNA. Denhardt's solution nevertheless has been utilised for all kinds of blot hybridizations, often at concentrations of 10 times that of the pre-incubation mix. Denhardt's solution, does not, however, consistently produce background-free hybridization and, reflecting efforts to overcome this problem, there have been many modifications to the original procedure. It is evident from this alone that the precise way in which non-specific DNA binding to nitrocellulose is reduced is unknown. In our experience clean, background-free, blot hybridization can sometimes be obtained in the absence of

ingredients in the annealing solution which are customarily included to control background. This fact suggests that the problem of non-specific autoradiographic background is not inherent in filter hybridization but probably originates in various impurities which increase the affinity of the probe for the membrane substrate.

The protocol described in the present paper uses heparin to reduce non-specific adsorbtion of nucleic acid probes to filters. The heparin effect may be based on the fact that it exhibits an essentially stochiometric binding affinity for DNA-binding proteins. This forms the basis of the use of heparin in the general heparin-agarose column chromatography method for purifying restriction endonucleases (3). Heparin will compete with DNA for protein binding and will inhibit the specific enzymatic functions of some DNA-binding proteins if added to a reaction before, but not after the DNA (4, 5). In this respect, therefore, heparin can be regarded as an analogue of DNA. The role of this heparin effect in controlling hybridization background probably lies in the fact that, whereas pure double-stranded DNA will not bind to nitrocellulose, the complex of DNA with certain proteins binds avidly to nitrocellulose. This effect is quite specific and has been used to investigate the precise nature of complex formation between DNA and site-specific endonucleases (5). Moreover, the more impure the preparation of restriction enzyme, the more DNA is bound (20), implying that the effect is not limited to restriction enzymes. The nitrocellulose binding is abolished by heparin if added before the enzyme is permitted to complex with the DNA. Under many conditions of hybridization there will be some renaturation to yield duplex radiolabelled DNA probe molecules. Heparin would be expected to form a complex with any substances which might alter the nitrocellulose binding properties of this proportion of the renatured DNA probe. Also, it cannot be ruled out that some impurities might influence the nature of binding between single-stranded nucleic acids and nitrocellulose under annealing conditions, so producing non-specific background effects. If the heparin effect does operate in respect of single-stranded as well as double-stranded DNA, the order in which the probe is added to the reaction relative to the heparin may be critical, but we have not investigated this point. In our procedure, heparin is present in the annealing mix before the probe is added. A further property of heparin, by analogy with single-stranded DNA, might include a binding capacity for nitrocellulose. If so it would also occupy sites on the filter membrane which normally adsorb DNA and its complexes but we have not investigated this possibility.

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